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First Inventor or Application Identifier

Fuminori TAKEMURA

Title

NUCLEIC ACID-BOUND POLYPEPTIDE, METHOD OF PRODUCING  
NUCLEIC ACID-BOUND POLYPEPTIDE, AND IMMUNOASSAY USING THE  
POLYPEPTIDE

**APPLICATION ELEMENTS**

See MPEP chapter 600 concerning utility patent application contents

ADDRESS TO:

Assistant Commissioner for Patents  
Box Patent Application  
Washington, DC 20231

1. ☒ Fee Transmittal Form (e.g. PTO/SB/17)  
(Submit an original and a duplicate for fee processing)

2. ☒ Specification

Total Pages **56**

3. ☒ Drawing(s) (35 U.S.C. 113) Total Sheets **2**

4. ☒ Oath or Declaration

Total Pages **3**

- a. ☐ Newly executed (original or copy)

- b. ☒ Copy from a prior application (37 C.F.R. §1.63(d))  
(for continuation/divisional with box 15 completed)

- i. ☐ DELETION OF INVENTOR(S)  
Signed statement attached deleting inventor(s) named  
in the prior application, see 37 C.F.R. §1.63(d)(2) and  
1.33(b).

5. ☒ Incorporation By Reference (usable if box 4B is checked)  
The entire disclosure of the prior application, from which a copy of the  
oath or declaration is supplied under Box 4B, is considered to be part  
of the disclosure of the accompanying application and is hereby  
incorporated by reference therein.

**ACCOMPANYING APPLICATION PARTS**

6. ☒ Application assigned to: FUJIREBIO INC.  
(Reel 8575, Frame 0118)

7. ☐ 37 C.F.R. §3.73(b) Statement ☐ Power of Attorney  
(when there is an assignee)

8. ☐ English Translation Document (if applicable)

9. ☒ Information Disclosure  
Statement (IDS)/PTO-1449 ☐ Copies of IDS  
Citations

10. ☐ Preliminary Amendment

11. ☒ White Advance Serial No. Postcard

12. ☐ Small Entity  
Statement(s) ☐ Statement filed in prior  
application. Status still proper  
and desired.

13. ☐ Certified Copy of Priority Document(s)  
(if foreign priority is claimed)

14. ☒ Other: REQUEST FOR PRIORITY

15. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below:

☐ Continuation ☒ Divisional ☐ Continuation-in-part (CIP) of prior application no.: 08/841,657

Prior application information: Examiner: HINES

Group Art Unit: 1641

16. Amend the specification by inserting before the first line the sentence:


☒ This application is a ☐ Continuation ☒ Division ☐ Continuation-in-part (CIP)  
of application Serial No. 08/841,657 Filed on APRIL 30, 1997

☐ This application claims priority of provisional application Serial No.

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**17. CORRESPONDENCE ADDRESS**

OBLON, SPIVAK, MCCLELLAND, MAIER & NEUSTADT, P.C.  
FOURTH FLOOR  
1755 JEFFERSON DAVIS HIGHWAY  
ARLINGTON, VIRGINIA 22202  
(703) 413-3000  
FACSIMILE: (703) 413-2220

Name:	Norman F. Oblon	Registration No.:	24,618
Signature:		Date:	5.7.99
Name:	Robert W. Hahl, Ph.D.	Registration No.:	33,893

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TITLE OF THE INVENTION

NUCLEIC ACID-BOUND POLYPEPTIDE,  
METHOD OF PRODUCING NUCLEIC ACID-BOUND POLYPEPTIDE,  
AND IMMUNOASSAY USING THE POLYPEPTIDE

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a nucleic acid-bound polypeptide, a method of producing the nucleic acid-bound polypeptide, and an immunoassay using the nucleic acid-bound polypeptide.

Discussion of Background

Various studies have been made as to how to maintain the specific steric structure of a combinant protein produced by gene engineering, more specifically gene manipulation, and also as to how to apply the thus produced protein to an antigen-antibody reaction.

In the production of the combinant protein, in particular, in the course of a purification step of the produced protein, a denaturation operation is inevitably carried out. In such purification step, it is not always possible to maintain a natural structure of the protein, so that such protein cannot be used in an immunoassay system.

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Various factors are also known that affect reactions which are peculiar to each of various assays. It is known that for these reasons or other, the above-mentioned antigen-antibody reaction does not always proceed as desired when the combinant protein is used.

For example, there is known an agglutination immunoassay as one of immunoassays. For instance, when an antibody corresponding to an antigen is assayed by agglutination immunoassay, the antigen is fixed on the surface of particles such as latex particles, and such antigen-fixed particles are allowed to react with the antibody in a test sample. When the antibody is present in the test sample, the antigen-fixed particles agglutinate due to the antigen-antibody reaction, so that, for instance, the absorbance of the test sample changes. Therefore by measuring the absorbance of the test sample, the degree of the agglutination can be determined, and accordingly the antibody in the test sample can be quantitatively measured from the measured absorbance of the test sample.

However, when the recombinant protein is used as the antigen to be fixed on the surface of the particles in the above-mentioned agglutination immunoassay, it occasionally occurs that even though the protein itself has reactivity with the antibody to be assayed and the antibody is in fact present in the test sample, no agglutination takes place.

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Conventionally, in the case where no agglutination takes place as mentioned above, the recombinant protein is modified or expressed in the form of a fused protein in order to improve the agglutination reactivity of the protein. However, it is extremely difficult to modify the protein so as to impart the desired properties thereto, while maintaining the antigenicity (i.e. the reactivity with the antibody).

Furthermore, the recombinant protein is often of an insoluble kind, so that when the thus produced protein is purified, the protein has to be subjected to solubilization treatment. However, the protein is often denatured in the course of the purification treatment, losing the necessary antigenicity.

Therefore, it is preferable that a soluble protein be directly produced by genetic engineering.

#### SUMMARY OF THE INVENTION

It is therefore a first object of the present invention to provide a modified polypeptide, which is modified so as to change the properties of polypeptide such as the isoelectric point, the molecular weight and the three-dimensional structure thereof, but without changing the antigenicity thereof.

A second object of the present invention is to provide a method of producing the above-mentioned recombinant polypeptide in such a manner that the

produced polypeptide can be obtained in a soluble fraction.

A third object of the present invention is to provide an immunoassay for assaying an antigen comprising a polypeptide, which is conventionally difficult to perform.

The first object of the present invention can be achieved by a nucleic acid-bound polypeptide which is produced by binding a nucleic acid to a polypeptide.

In the above nucleic acid-bound polypeptide, the nucleic acid may be bound to at least one terminus of the polypeptide.

The nucleic acid-bound polypeptide may further comprise a nucleic acid-binding motif through which the nucleic acid is bound to the polypeptide.

The above-mentioned polypeptide and the nucleic acid-binding motif may be expressed in the form of a fusion polypeptide by genetic engineering.

The nucleic acid-binding motif may have an amino acid sequence with sequence No. 2 defined in a sequence table attached to the specification of this application.

The above-mentioned polypeptide can be used as an antigen to be assayed by an immunoassay.

The second object of the present invention can be achieved by a method of producing a nucleic acid-bound polypeptide comprising the steps of:

producing a recombinant polypeptide,

binding a nucleic acid to the recombinant polypeptide to produce a nucleic acid-bound polypeptide as a soluble fraction, and

purifying the nucleic acid-bound polypeptide from the soluble fraction.

In the above-mentioned method of producing the nucleic acid-bound polypeptide, the step of binding the nucleic acid to the polypeptide to produce the nucleic acid-bound polypeptide may comprises the steps of:

fusing a gene which codes the polypeptide and a gene which codes the nucleic acid-binding motif to produce a fusion gene, and

expressing the fusion gene to produce the nucleic acid-bound polypeptide via the nucleic acid-binding motif.

The third object of the present invention can be achieved by an immunoassay for assaying an antigen comprising a polypeptide, or an antibody corresponding to the polypeptide, using as an antigen for the immunoassay the above-mentioned nucleic acid-bound polypeptide.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings,

wherein:

Fig. 1 is a genetic map of a cloning vector pW6A for use in expressing HCV core protein used in examples of the present invention.

Fig. 2 is a diagram showing the results of Western blotting performed for showing the reactivity of a HCV core protein prepared by genetic engineering in an example of the present invention with HCV core positive human serum.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

The nucleic acid-bound polypeptide of the present invention can be provided by binding a nucleic acid to a polypeptide, whereby the properties of the polypeptide, such as the isoelectric point, the molecular weight and the three-dimensional structure thereof, can be changed without the antigenicity thereof being changed.

As the "polypeptide" for use in the present invention, any polypeptide can be employed as long as the polypeptide itself exhibits antigenicity and therefore the number of amino acid residues which constitute the polypeptide is 6 or more. It is preferable that the number of the amino acid residues which constitute the "polypeptide" for use in the present invention be 8 or more.

Examples of the "polypeptide" for use in the present invention include composites of a polypeptide and another

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component or other components such as sugar or lipid, namely glycoprotein and lipo protein.

There is no particular limitation to the size of the nucleic acid which is bound to the polypeptide as long as the nucleic acid can change the above-mentioned properties of the polypeptide, such as isoelectric point, molecular weight and three-dimensional structure, without changing the antigenicity thereof. Normally, the number of bases of the nucleic acid for use in the present invention is 100 b to 10 kb, preferably about 1 kb to 5 kb.

Furthermore, the nucleic acid to be bound to the polypeptide may be either DNA or RNA. In the present invention, there is no limitation to the nucleotide sequence to be bound to the polypeptide. Any nucleotide sequence is acceptable for use in the present invention.

The nucleic acid may be bonded to any portion of the polypeptide. For instance, the nucleic acid may be bonded to the N-terminus or the C-terminus of the polypeptide, but the bonding is not limited to such terminus. In the present invention, the nucleic acid may be either directly or indirectly bonded to the polypeptide. For instance, the nucleic acid may be bonded to the polypeptide via a nucleic acid-binding motif which is also a polypeptide.

In this application, with respect to the binding of the nucleic acid to the polypeptide, the term "binding"



or "bound" means all kinds of chemical bondings between the polypeptide and the nucleic acid with attractive force in a wide range of relatively weak attractive force to strong attractive force, without any particular limitation to the bonding mode, including the so-called association, covalent bonding, ionic bonding, coordinate bonding, and hydrogen bonding.

In the present invention, when the nucleic acid-bound polypeptide is produced by genetic engineering, the nucleic acid-bound polypeptide may be expressed in the form of a polypeptide to which the nucleic acid is bound, thereby producing the nucleic acid-bound polypeptide. Alternatively, after a recombinant polypeptide is expressed, the nucleic acid may be bound to the recombinant polypeptide, thereby producing the nucleic acid-bound polypeptide.

To be more specific, when a polypeptide is expressed as a fusion polypeptide, with a nucleic acid-binding motif which is known to have a function of binding the nucleic acid to the polypeptide being included in the function of the polypeptide to be expressed, a polypeptide with the nucleic acid-binding motif is expressed, and the nucleic acid in the host is simultaneously bound to the recombinant polypeptide via the nucleic acid-binding motif, so that the nucleic acid-bound polypeptide can be produced. This nucleic acid-bound polypeptide can be purified thereafter.

Alternatively, the nucleic acid-bound polypeptide can be obtained by reconstituting the polypeptide by mixing the expressed polypeptide with the nucleic acid.

In connection with the above-mentioned nucleic acid-binding motif, various nucleic acid-binding motifs are known. For example, in J. of Virology, 64 3319-3330 (1990), there is reported a nucleic acid-binding motif which is present in HBc protein amino acid sequence of hepatitis B virus (HBV), and in Biochim. Biophys. Act, 950, 45-53 (1988), there is reported protamin, which is a nucleic acid-bound protein in mouse. These can also be employed in the present invention.

The nucleotide sequence and the amino acid sequence of the nucleic acid-binding motif of HBc are respectively shown in the sequence No. 1 and the sequence No. 2 in the sequence table attached to this specification; and the nucleotide sequence and the amino acid sequence of the mouse protamine are respectively shown in the sequence No. 17 and the sequence No. 18 in the sequence table attached to this specification

As mentioned above, when the protein or polypeptide conventionally produced by genetic engineering is used as the antigen to be fixed on the surface of the particles in the conventional agglutination immunoassay, it occasionally occurs that even though the polypeptide itself has reactivity with the antibody to be assayed and the antibody is in fact present in the test sample, no

agglutination takes place.

In the present invention, however, this conventional problem is completely solved by use of the nucleic acid-bound polypeptide. Namely, when the nucleic acid-bound polypeptide of the present invention is used as the antigen to be fixed on the surface of particles for use in the agglutination immunoassay, the agglutination successfully takes place proportionally in accordance with the amount of the corresponding antibody in the test sample.

The nucleic acid-bound polypeptide of the present invention can be applied not only to the above-mentioned agglutination, but also to any conventional immunoassay such as ELISA (enzyme-linked immunosorbent assay).

In the present invention, when the antibody in a test sample is assayed, the nucleic acid-bound polypeptide may be fixed as the corresponding antigen, for example, on the surface of solid particles and is allowed to react with the antibody in the test sample, so that the amount of the antibody in the test sample is measured as corresponding to the amount of the antibody which has reacted with the nucleic acid-bound polypeptide fixed as the corresponding antigen on the surface of solid particles.

Furthermore, in the present invention, an antigen comprising a polypeptide can also be assayed. In this

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assay system, the nucleic acid-bound polypeptide may also be fixed, for example, on the surface of solid particles. An antigen in the test sample comprises the same polypeptide as in the nucleic acid-bound polypeptide.

In this assay system, the nucleic acid-bound polypeptide fixed on the solid particles and the antigen in the test sample are both present as antigens. When an antibody corresponding to the antigen in the test sample is added in the form of an antibody agent to this assay system, there occur competition antigen/antibody reactions between the antigen in the test sample and the antibody in the agent, and between the nucleic acid-bound polypeptide fixed on the solid particles and the antibody in the agent. In other words, the reaction between the nucleic acid-bound polypeptide and the antibody in the agent is hindered to some degree by the reaction between the antigen in the test sample and the antibody in the agent, namely by the presence of the antigen in the test sample. From the degree of the reaction hindrance, the amount of the antigen in the test sample can be assayed.

Thus, the polypeptide antigen in a test sample can also be assayed by carrying out the above-mentioned competition reaction with the addition of a known amount of the nucleic acid-bound polypeptide to the test sample.

Conventionally, when polypeptide is produced by

genetic engineering, in many cases, the recombinant polypeptide is obtained as an insoluble fraction. Therefore, when the thus obtained polypeptide is used in practice, the polypeptide must be subjected to solubilization treatment. However, the polypeptide is often denatured in the course of the solubilization treatment, changing the antigenicity. Therefore it is preferable that the recombinant polypeptide be obtained as a soluble fraction.

In the method of producing the nucleic acid-bound polypeptide of the present invention, for example, a polypeptide is produced by genetic engineering, and the thus produced polypeptide is simultaneously caused to be bound to a nucleic acid in the host, whereby the nucleic acid-bound polypeptide is obtained as a soluble fraction.

Furthermore, as shown in the following examples, for example, when the polypeptide to be expressed is expressed as a fused polypeptide of a polypeptide and a nucleic acid-binding motif of HBc, the nucleic acid is bound to the nucleic acid-binding motif at the same time as the expression thereof, so that the nucleic acid-bound polypeptide is obtained in the soluble fraction.

Thus, there can be attained the method of producing the nucleic acid-bound polypeptide of the present invention, which comprises the steps of producing the recombinant polypeptide, binding the nucleic acid to the polypeptide to produce the nucleic acid-bound polypeptide

as a soluble fraction, and purifying the nucleic acid-bound polypeptide from the soluble fraction.

Other features of this invention will become apparent in the course of the following description of exemplary embodiments, which are given for illustration of the invention and are not intended to be limiting thereof.

#### Reference Example 1

##### [Expression of HCV Core Protein (1 - 120aa)]

A DNA fragment for coding the HCV core polypeptide with sequence ID. No. 3 in the attached sequence table was amplified by the PCR (Polymerase Chain Reaction) method, using as a template molecule a plasmid CKSC1150 with a DNA fragment including an HCV core region, and was then digested with a restriction endonuclease EcoRI and a restriction endonuclease BamHI.

An HCV core region-including DNA fragment 370 bp was separated by 1% agarose gel electrophoresis. This DNA fragment was inserted into an EcoRI - BamHI site of an expression plasmid pW6A shown in Fig. 1, so that a plasmid pW6AHCV core 120 was prepared.

By use of this plasmid, *Escherichia coli* BL21 (DE3) (obtained from Brookhaven National Laboratory) was subjected to transformation, so that an ampicillin-resistant transformed *Escherichia coli* BL21 (DE3)/pW6AHCV

core 120 for expressing HCV core polypeptide 120 was obtained, and an HCV core protein (1 - 120 aa) was expressed. Hereinafter, the thus expressed protein is referred to as "120". The nucleotide sequence of "120" and the amino acid sequence of "120" are respectively shown in Sequence ID. No. 3 and Sequence ID. No. 4 in the sequence table attached to this specification.

#### **Example 1**

##### **[Preparation of Plasmid]**

A DNA fragment for coding HCV core polypeptides 150 and 120 which are respectively shown with sequence ID. No. 5 and with sequence ID. No. 3 in the attached sequence table was amplified by the PCR (Polymerase Chain Reaction) method, using as a template molecule a plasmid CKSC1150 with a DNA fragment including a HCV core region being introduced, and was then digested with a restriction endonuclease EcoRI and a restriction endonuclease BamHI.

An HCV core region-including DNA fragment 470 bp and an HCV core region-including DNA fragment 370 bp were separated by 1% agarose gel electrophoresis. These DNA fragments were inserted into an EcoRI - BamHI site of the expression plasmid pW6A shown in Fig. 1, whereby a plasmid pW6AHCV core 150 and a plasmid pW6AHCV core 120 were prepared.

A DNA fragment for coding an HBc nucleic acid-binding motif shown with sequence ID. No. 1 in the sequence table attached to this specification was amplified by the PCR (Polymerase Chain Reaction) method, using as a template molecule a plasmid pHBV-11 (Nucleic Acids Res., 18, 4587 (1990)), and was then digested with the BamHI.

A DNA fragment 110bp including a nucleic acid-binding motif was separated by 2% agarose gel electrophoresis. This DNA fragment was inserted into an EcoRI - BamHI site of each of the above-mentioned plasmid pW6AHCV core 150 and plasmid pW6AHCV core 120.

By use of these plasmids, *Escherichia coli* BL21 (DE3) (obtained from Brookhaven National Laboratory) was subjected to transformation, so that an ampicillin-resistant transformed *Escherichia coli* BL21 (DE3)/pW6AHCV core 150NA and an ampicillin-resistant transformed *Escherichia coli* BL21 (DE3)/pW6AHCV core 120NA were obtained.

In this specification, the proteins to which the nucleic acid-binding motif is bound for expressing the above-mentioned transformed *Escherichia coli* BL21 (DE3)/pW6AHCV core 150NA and the above-mentioned transformed *Escherichia coli* BL21 (DE3)/pW6AHCV core 120NA are respectively referred to as "150NA" and "120NA".



The nucleotide sequence of "150NA" and the amino acid sequence of "150NA" are respectively shown in Sequence ID. No. 9 and Sequence ID. No. 10 in the sequence table attached to this specification; and the nucleotide sequence of "120NA" and the amino acid sequence of "120NA" are respectively shown in Sequence ID. No. 7 and Sequence ID. No. 8 in the sequence table attached to this specification.

#### Example 2

##### [Expression of Recombinant Protein (150NA and 120NA)]

Each of the transformed *Escherichia coli* BL21 (DE3)/pW6AHCV core 150 and the transformed *Escherichia coli* BL21 (DE3)/pW6AHCV core 120 prepared in Example 1 was separately cultured overnight in 2 ml of an LB culture medium containing 50 µg/ml of ampicillin at 37°C.

After the optical density (OD) of each culture medium reached to 0.6 to 0.8 with a light with a wavelength of 600 nm by preculture, expression induction was carried out with the addition of 0.5 mM IPTG (Isopropyl-β-D(-)-thiogalactopyranoside) thereto, and the cultivation was continued for another two hours.

1.5 ml of the *Escherichia coli* cultivation medium was centrifuged at 5000 rpm for 2 minutes, whereby the *Escherichia coli* was collected. The thus collected

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*Escherichia coli* was suspended in 100  $\mu$ l of a buffer solution (10 mM tris-HCl, pH 8.0, 0.1 M NaCl, 1 mM EDTA), and was then subjected to ultrasonic disruption for 15 minutes, whereby the *Escherichia coli* was completely disrupted, whereby two test samples, namely an *Escherichia coli* test sample of *Escherichia coli* BL21 (DE3)/pW6AHCV core 150NA and an *Escherichia coli* test sample of *Escherichia coli* BL21 (DE3)/pW6AHCV core 120NA, were prepared.

8  $\mu$ l of a three-times concentration SDS polyacrylamide buffer solution (0.15 M tris-HCl, pH 6.8, 6% SDS, 24% glycerol, 6 mM EDTA, 2% 2-mercaptoethanol, 0.003% bromophenol blue) was added to each of the above test samples separately. Each mixture was then stirred sufficiently and was subjected to SDS-polyacrylamide gel electrophoresis.

Western blotting was performed on a nitrocellulose filter, using each of the thus prepared test samples. After performing blocking using 1% BSA, each of the test samples was allowed to react with an HCV core antibody human serum which was diluted 200 times with a phosphoric acid buffer solution (10 mM phosphoric acid, pH 7.4, 0.15 M NaCl). Furthermore, a peroxylase enzyme labeled anti-human IgG rabbit polyclonal antibody (made by Dako Co., Ltd.) was then allowed to react therewith. After washing, 10 ml of a substrate coloring liquid (0.01 %

aqueous solution of hydrogen peroxide, 0.6 mg/ml 4-chloro-1-naphthol) was added thereto, whereby each test sample was colored.

The results are shown in Fig. 2. As shown in Fig. 2, both the *Escherichia coli* test sample of *Escherichia coli* BL21 (DE3)/pW6AHCV core 150NA and the *Escherichia coli* test sample of *Escherichia coli* BL21 (DE3)/pW6AHCV core 120NA exhibited a positive reaction with the HCV core antibody human serum.

### Example 3

#### [Purification of Soluble Nucleic Acid-bound 120NA Recombinant Protein (120(+))]

The *Escherichia coli* BL21 (DE3)/pW6AHCV core 120NA prepared in Example 1 was cultured overnight in an LB culture medium at 37°C. The optical density (OD) of the culture medium was adjusted by preculture so as to be about 0.7 when measured with light with a wavelength of 600 nm. Expression induction was then carried out with the addition of 0.5 mM IPTG thereto, and thereafter the cultivation was continued for two hours and 30 minutes.

The *Escherichia coli* cultivation medium was centrifuged, whereby the *Escherichia coli* was collected. To the thus collected *Escherichia coli*, 150 ml of a buffer solution (50 mM tris-HCl, pH 8.0, 20% ethanol,

0.2 M NaCl, 0.3% octylthioglucoside (hereinafter referred to as "OTG") was added, and the mixture was ice-cooled and subjected to ultrasonic disruption.

This mixture was then centrifuged, whereby a soluble fraction which contained therein a nucleic acid-bound 120NA (hereinafter referred to as "120NA(+)") was recovered.

A 50%-sucrose concentration buffer solution was prepared by adding sugar to a buffer solution (50 mM tris-HCl, pH 8.0, 20% ethanol) in such a manner that the concentration of sucrose in the buffer solution was 50%. In the same manner as mentioned above, a 30%-sucrose concentration buffer solution, and a 20%-sucrose concentration buffer solution were prepared.

These buffer solutions were overlaid in an ultracentrifuge tube in the direction from the bottom to the top portion of the tube in the order of the 50%-sucrose concentration buffer solution, the 30%-sucrose concentration buffer solution and the 20%-sucrose concentration buffer solution.

The 120NA(+) containing soluble fraction was overlaid on top of the overlaid buffer solutions in the ultracentrifuge tube, and was then subjected to a first sucrose density gradient ultracentrifugation at 10°C, with a centrifugal force of 100,000 g, for 12 hours, using a Beckman ultrasonic centrifuge.

The 120NA(+) was recovered in a portion with a

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sucrose concentration of about 30 to 40%.

The 120NA(+) containing fraction recovered by the first sucrose density gradient ultracentrifugation was purified by Superdex 200 (gel filtration column) (made by Pharmacia Co., Ltd.) which was equilibrated with a buffer solution (0.3 M NaCl, 0.1% myristyl sulfobetaine (Trademark "SB3-14" made by Sigma Co., Ltd.), whereby 120NA(+) with a molecular weight of about 700 to 1000 kDa was recovered.

A 50%-sucrose concentration buffer solution was prepared by adding sucrose to a buffer solution (50 mM tris-HCl, pH 8.0, 20% ethanol) in such a manner that the concentration of sucrose in the buffer solution was 50%. In the same manner as mentioned above, a 20%-sucrose concentration buffer solution was prepared.

These buffer solutions were overlaid in an ultracentrifuge tube in the direction from the bottom to the top portion of the tube in the order of the 50%-sucrose concentration buffer solution and the 20%-sucrose concentration buffer solution.

The above-mentioned 120NA(+) with a molecular weight of about 700 to 1000 kDa was overlaid on top of the overlaid buffer solutions in the ultracentrifuge tube, and was then subjected to a second sucrose density gradient ultracentrifugation at 10°C, with a centrifugal force of 100,000 g, for 12 hours, using the Beckman ultrasonic centrifuge, whereby the 120NA(+) was

concentrated and purified.

## Reference Example 2

### [Purification of Insoluble 120NA]

The *Escherichia coli* BL21 (DE3)/pW6AHCV core 120NA prepared in Example 1 was cultured overnight in an LB culture medium at 37°C. The optical density (OD) of the culture medium was adjusted by preculture so as to have about 0.7 when measured with light with a wavelength of 600 nm. Expression induction was then carried out with the addition of 0.5 mM IPTG thereto, and the cultivation was continued for two hours and 30 minutes.

The *Escherichia coli* cultivation medium was then centrifuged, whereby the *Escherichia coli* was collected. To the thus collected *Escherichia coli*, 150 ml of a buffer solution (50 mM tris-HCl, pH 8.0, 20% ethanol, 0.2 M NaCl, 0.3% OTG) was added, and the mixture was ice-cooled and subjected to ultrasonic disruption.

This mixture was then centrifuged, whereby an insoluble 120NA fraction was obtained. The thus obtained insoluble 120NA fraction was made soluble by a buffer solution (6M urea, 50 mM glycine-NaOH, pH 11.7) and was then subjected to centrifugation, whereby a supernatant fraction was obtained.

The thus obtained supernatant fraction was subjected to ion exchange purification, using an SFF cationic ion

exchange column (made by Pharmacia Co., Ltd.) which was equilibrated with a buffer solution (6M urea, 50 mM glycine-NaOH, pH 11.7), with sodium chloride elution.

The SFF eluted fraction was then purified, using Superdex 200 (gel filtration column) (made by Pharmacia Co., Ltd.) which was equilibrated with a buffer solution (6M urea-0.5M NaOH, 50 mM tris-HCl, pH 9.6). Thus, a purified 120NA was obtained in a portion with a molecular weight of about 22 kDa.

#### **Example 4**

##### **[Confirmation of Properties of 120NA and 120NA (+)]**

The OD 260/280 nm ratio of the 120NA(+) purified in Example 3 was measured. The result was that the OD 260/280 nm ratio of the 120NA(+) was about 2.0, which was greater than the OD 260/280 nm ratio of the 120NA. This indicated that at least the polypeptide and the nucleic acid coexist in the 120NA(+).

Furthermore, in the sucrose density gradient ultracentrifugation, the 120NA was mostly collected in the zero% sucrose concentration region, while the 120NA(+) was mostly collected in an about 30-40% sucrose concentration region. It is considered that this fact indicates that the density of the 120NA(+) is different from that of the 120NA.

The 120NA(+) was subjected to enzyme treatment,

using DNase or RNase. When the 120NA(+) was subjected to enzyme treatment, using RNase, the nucleic acid contained in the 120NA(+) was decomposed in its entirety by the RNase. It is considered that this fact indicates that the constituent nucleic acid of the 120NA(+) is RNA.

The 120NA(+) was also subjected to isoelectric focusing. The isoelectric point of the 120NA(+) was present in a wide range of pI 3.5 to 5.0.

In sharp contrast to this, the isoelectric points of the 120NA purified in Reference Example 2 was pI 12.84, with a strong positive charge, which was significantly different from the isoelectric point of the 120NA(+).

Furthermore, the 120NA(+) was also subjected to Native electrophoresis, using a 3% agarose 3% polyacrylamide gel. From the fact that luminescence was observed at the time of Ethidium bromide stain of the 120NA(+), it was confirmed that the nucleic acid was contained in the 120NA(+).

The 120NA(+) was further subjected to Western blotting and Coomassie Brilliant Blue stain, using the same gel as used in the above-mentioned Ethidium Bromide stain. The result was that in the Western blotting, the reactivity of the 120NA(+) with an anti-HCV core antibody was observed at the same position as that of the portion made luminescent by the Ethidium Bromide stain; and in the Coomassie Brilliant Blue stain, the presence of the polypeptide was confirmed.



In sharp contrast to this, with respect to the 120A, the transfer of the 120NA into the gel was not confirmed in the Native electrophoresis even when the Western blotting and the Coomassie Brilliant Blue stain were carried out.

Thus, the properties of the 120NA(+) are entirely different from those of the 120NA with respect to the apparent molecular weight, the density, and the electric charge thereof, particularly because of the increase of the apparent molecular weight of the 120NA(+) due to the binding of the nucleic acid to the polypeptide in the 120NA(+), but there are no differences in the Western blotting and agglutination reactions between the two. From these facts, it is considered that the antigenicity is maintained in the 120NA(+).

### Reference Example 3

#### [Expression of Lysine-fused 120 (120K10)]

In the same manner as in Example 1, pW6AHCV core 120 was subjected to such gene manipulation that 10 lysine residues were continuously fused to the C-terminus of pW6AHCV core 120, whereby pW6AHCV core 120K10 was prepared.

By use of this pW6AHCV core 120K10, *Escherichia coli* BL21 (DE3) was subjected to transformation, whereby an ampicillin-resistant transformed *Escherichia coli* BL21

(DE3)/pW6AHCV core K10 was obtained. Hereinafter, the protein expressed by this ampicillin-resistant transformed *Escherichia coli* BL21 (DE3)/pW6AHCV core K10 is referred to as 120K10.

The above transformed *Escherichia coli* BL21 (DE3)/pW6AHCV core K10 was cultured overnight in an LB culture medium at 37°C. The optical density (OD) of the culture medium was adjusted by preculture so as to have about 0.7 when measured with light with a wavelength of 600 nm. Expression induction was then carried out with the addition of 0.5 mM IPTG thereto, and the cultivation was continued for two hours and 30 minutes.

The *Escherichia coli* cultivation medium then was centrifuged, whereby the *Escherichia coli* was collected. To the thus collected *Escherichia coli*, 150 ml of a buffer solution (50 mM tris-HCl, pH 8.0, 20% ethanol, 0.2 M NaCl, 0.3% OTG) was added, and the mixture was ice-cooled and subjected to ultrasonic disruption.

This mixture was then centrifuged, whereby a soluble 120K10 fraction and an insoluble 120K10 fraction were separately obtained.

A 50%-sucrose concentration buffer solution was prepared by adding sucrose to a buffer solution (50 mM tris-HCl, pH 8.0, 20% ethanol) in such a manner that the concentration of sucrose in the buffer solution was 50%. In the same manner as mentioned above, a 30%-sucrose

concentration buffer solution and a 20%-sucrose concentration buffer solution were prepared.

These buffer solutions were overlaid in an ultracentrifuge tube in the direction from the bottom to the top portion of the tube in the order of the 50%-sucrose concentration buffer solution, the 30%-sucrose concentration buffer solution and the 20%-sucrose concentration buffer solution.

The above-mentioned soluble 120K10 fraction was overlaid on top of the overlaid buffer solutions in the ultracentrifuge tube, and was then subjected to a sucrose density gradient ultracentrifugation at 10°C, with a centrifugal force of 100,000 g, for 12 hours, using the Beckman ultrasonic centrifuge. The 120K10 was not recovered in any of the 50%-sucrose concentration buffer solution, the 30%-sucrose concentration buffer solution and the 20%-sucrose concentration buffer solution, but was recovered on the top layer portion in the tube.

The above-mentioned insoluble 120K10 fraction was purified in the same manner as in Reference Example 2, using the SFF cationic ion exchange column (made by Pharmacia Co., Ltd.) and performing the gel filtration, whereby a purified 120K10 was recovered in a portion with a molecular weight of about 20 kDa.

#### **Reference Example 4**

**[Assay of HCV Core Antigen Positive Serum]**

The reactivity of each of HCV antibody positive serum 1 and HCV antibody positive serum 2 with a commercially available HCV antibody assay agent (Trademark "RIBA HCV 3.0 STRIP IMMUNOBLOT ASSAY" made by Chiron Co., Ltd.), using HCV antigen c100 (Amino acid Nos. 1569-1931), HCV antigen c33c (Amino acid Nos. 1192-1457), core antigen c22 (Amino acid Nos. 2-120) and NS5 (Amino acid Nos. 2054-2995). The result was that both HCV antibody positive serum 1 and HCV antibody positive serum 2 have antibodies in the entire antigen region including the core antigen region.

TABLE 1

Reactivity Tests of Positive Serums

	c100	c33c	Core Antigen	NS5	Judgement
Positive Serum 1	4+	4+	4+	4+	Positive
Positive Serum 2	4+	4+	4+	4+	Positive

#### Example 5

Each of the HCV antigens obtained in Reference Examples 1, 2, 3 and Example 3 was fixed on the surface of gelatin particles (made by Fujirebio Co., Ltd.) with a concentration of 10 mg/ml in a buffer solution (0.15M PBS, pH 7.1).

By use of HCV antibody positive serum 1 and HCV

antibody positive serum 2 confirmed as having antibodies in the entire antigen region including the core antigen region in Reference Example 4, and a monoclonal antibody #2-7 obtained by subjecting HCV core antigen c22 to immunization, the immune reactivity of each of the above-mentioned HCV antigens fixed on the surface of gelatin particles was investigated.

25  $\mu$ l of each HCV antigen-fixed gelatin particles and 25  $\mu$ l of one of the above-mentioned HCV antibody positive serum 1 or HCV antibody positive serum 2, or 25  $\mu$ l of the monoclonal antibody #2-7 were allowed to react in a microtiter plate (made by Fujirebio Co., Ltd.) for 2 hours, and agglutination images thereof were investigated. The results are shown in TABLE 2. In TABLE 2, the reactivity is shown with a dilution rate of 2<sup>n</sup>, and when a positive agglutination image was observed even when n was 4 or more in the dilution rate, the immune reactivity was judged as being "positive".

The monoclonal antibody #2-7 obtained by subjecting HCV core antigen c22 to immunization reacted with any HCV core antigen, but it was only the 120NA(+) fixed gelatin particles that HCV antibody positive serum 1 and HCV antibody positive serum 2 reacted in the above-mentioned reactions.

TABLE 2

Immune Reactivity Tests of HCV Core Antigens

Name of Core Antigen	Positive Serum 1	Positive Serum 2	#2-7
120NA(+)	6+	7	8
120NA	<3	<3	7
120K10	<3	<3	6
120	<3	<3	4

#### Example 6

##### [Rearrangement of 120NA(+) from 120NA]

By use of the transformed *Escherichia coli* BL21 (DE3)/pW6AHCV core 120NA prepared in Example 1, HCV core 120NA was purified from an insoluble fraction thereof in the same manner as in Reference Example 2. The molecular weight of the purified HCV core 120NA was about 22 kDa, and the OD 260/280 nm ratio thereof was about 0.7.

To the HCV core 120NA (hereinafter referred to as 120NA), a cyclic plasmid DNA (4.7 Kbp) derived from pW6A, 6M urea and 20% sucrose were added, and 120NA was dialyzed against a buffer solution (50 mM tris-HCL, 0.15M NaCl, 20% sucrose), whereby 120NA was rearranged to 120NA(+).

The 120NA(+) which was obtained by the above-mentioned dialysis and rearrangement was purified, using Superdex 200 (gel filtration column) (made by Pharmacia

Co., Ltd.), whereby the 120NA(+) was recovered in a portion with a molecular weight of 700 to 1000 kDa.

A 50%-sucrose concentration buffer solution was prepared by adding sucrose to a buffer solution (50 mM tris-HCl, pH 8.0, 20% ethanol) in such a manner that the concentration of sucrose in the buffer solution was 50%. In the same manner as mentioned above, a 20%-sucrose concentration buffer solution was prepared.

These buffer solutions were overlaid in an ultracentrifuge tube in the direction from the bottom to the top portion of the tube in the order of the 50%-sucrose concentration buffer solution and the 20%-sucrose concentration buffer solution.

The above recovered 120NA(+) was overlaid on top of the overlaid buffer solutions in the ultracentrifuge tube, and was then subjected to a sucrose density gradient ultracentrifugation at 10°C, with a centrifugal force of 100,000 g, for 12 hours, using the Beckman ultrasonic centrifuge. The rearranged 120NA(+) was recovered in an about 40% to 50%-sucrose concentration portion of the buffer solution.

The OD 260/280 nm ratio of the 120NA before the rearrangement was about 0.7, and when the 120NA was rearranged to the 120NA(+), the OD 260/280 nm ratio thereof was changed from about 0.7 to about 1.7.

Furthermore, the above-mentioned rearranged 120NA(+) and the soluble 120NA(+) prepared in Example 3

have almost the same molecular weight after the gel filtration thereof, and also have almost the same specific weight thereof after the sucrose density gradient ultracentrifugation thereof. Thus, it is considered that these facts indicate that the above-mentioned rearrangement from the 120NA to the 120NA(+) was successfully conducted.

#### **Example 7**

**[Construction of Transformed *Escherichia coli* BL21 (DE3)/pW6AHCV Core 120NA120 for Expressing 120-fused 120NA (120NA120)]**

A DNA fragment for coding an HCV core polypeptide shown with sequence ID. No. 3 in the attached sequence table was amplified by the PCR method, using as a template molecule a plasmid CKSC1150 with a DNA fragment including a HCV core region being introduced, and was then digested with a restriction endonuclease *NheI* and a restriction endonuclease *EcoRI*.

An HCV core region-including DNA fragment 370 bp was separated by 1% agarose gel electrophoresis. This DNA fragment was inserted into an *NheI* - *EcoRI* site of the expression plasmid pW6A shown in Fig. 1, whereby a plasmid pW6AHCV core 120 (*NheI*/*EcoRI*) was prepared.

A DNA fragment for coding the HCV core polypeptide shown with sequence ID. No. 3 in the attached sequence table was amplified by the PCR method, using as a



template molecule a plasmid CKSC1150, and was then digested with a restriction endonuclease EcoRI and a restriction endonuclease BamHI.

An HCV core region-including DNA fragment 370 bp was then separated by 1% agarose gel electrophoresis. This DNA fragment was inserted into an EcoRI - BamHI site of the plasmid pW6AHCV core 120 (NheI/EcoRI), whereby a plasmid pW6AHCV core 120-120 was prepared.

A DNA fragment for coding an HBc nucleic acid-binding motif with sequence ID. No. 1 in the attached sequence table was amplified by the PCR method, using as a template molecule a plasmid pHBV-11, and was then digested with a restriction endonuclease EcoRI.

A DNA fragment 110bp including the nucleic acid-binding motif was separated by 2% agarose gel electrophoresis. This DNA fragment was inserted into an EcoRI site of the above-mentioned plasmid pW6AHCV core 120-120.

By use of this plasmid, *Escherichia coli* BL21 (DE3) was subjected to transformation, so that an ampicillin-resistant transformed *Escherichia coli* BL21 (DE3)/pW6AHCV core 120NA120 for expressing 120-fused 120NA (hereinafter referred to as 120NA120) was obtained.

#### Example 8

##### [Purification of Insoluble 120NA120]

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In the same manner as in Reference Example 2, the transformed *Escherichia coli* BL21 (DE3)/pW6AHCV core 120NA120 prepared in Example 7 was cultured overnight in an LB culture medium at 37°C. The optical density (OD) of the culture medium was adjusted by preculture so as to have about 0.7 when measured with light with a wavelength of 600 nm. Expression induction was then carried out with the addition of 0.5 mM IPTG thereto, and the cultivation was continued for two hours and 30 minutes.

The *Escherichia coli* cultivation medium was then centrifuged, whereby the *Escherichia coli* was collected. To the thus collected *Escherichia coli*, 150 ml of a buffer solution (50 mM tris-HCl, pH 8.0, 20% ethanol, 0.2 M NaCl, 0.3% OTG) was added, and the mixture was ice-cooled and subjected to ultrasonic disruption.

This mixture was then centrifuged, whereby an expressed 120NA120 was obtained as a soluble fraction as well as an insoluble fraction. The insoluble 120NA120 fraction was made soluble by a buffer solution (6M urea, 50 mM glycine-NaOH, pH 11.0) and was then subjected to centrifugation, whereby a supernatant fraction was obtained.

The thus obtained supernatant fraction was subjected to ion exchange purification, using an SFF cationic ion exchange column (made by Pharmacia Co., Ltd.) which was equilibrated with a buffer solution (6M urea-glycine-

NaOH, pH 11.0), with sodium chloride elution. 120NA120 was recovered in an about 0.5M sodium chloride elution fraction.

The SFF eluted fraction was then purified, using Superdex 200 (gel filtration column) (made by Pharmacia Co., Ltd.) which was equilibrated with a buffer solution (6M urea-0.5M NaCl, 50 mM tris-HCl, pH 9.6). Thus, a purified 120NA120 was obtained in a portion with a molecular weight of about 40 kDa.

The nucleotide sequence and the amino acid sequence of the 120NA120 are respectively shown with Sequence ID. No. 11 and Sequence ID. No. 12 in the attached sequence table.

#### **Example 9**

##### **[Purification of Soluble Nucleic Acid-bound 120NA120 (120NA120(+))]**

In the same manner as in Example 3, the *Escherichia coli* BL21 (DE3)/pW6AHCV core 120NA120 prepared in Example 7 was cultured overnight in an LB culture medium at 37°C. The optical density (OD) of the culture medium was adjusted by preculture so as to be about 0.7 when measured with light with a wavelength of 600 nm. Expression induction was then carried out with the addition of 0.5 mM IPTG thereto, and thereafter the cultivation was continued for two hours and 30 minutes.

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The *Escherichia coli* cultivation medium was centrifuged, whereby the *Escherichia coli* was collected. To the thus collected *Escherichia coli*, 150 ml of a buffer solution (50 mM tris-HCl, pH 8.0, 20% ethanol, 0.2 M NaCl, 0.3% OTG) was added, and the mixture was ice-cooled and subjected to ultrasonic disruption.

This mixture was then centrifuged, whereby a soluble nucleic acid-bound 120NA120 (hereinafter referred to as "120NA120(+)") was recovered.

A 50%-sucrose concentration buffer solution was prepared by adding sucrose to a buffer solution (50 mM tris-HCl, pH 8.0, 20% ethanol) in such a manner that the concentration of sucrose in the buffer solution was 50%. In the same manner as mentioned above, a 30%-sucrose concentration buffer solution, and a 20%-sucrose concentration buffer solution were prepared.

These buffer solutions were overlaid in an ultracentrifuge tube in the direction from the bottom to the top portion of the tube in the order of the 50%-sucrose concentration buffer solution, the 30%-sucrose concentration buffer solution and the 20%-sucrose concentration buffer solution.

The 120NA120(+) containing soluble fraction was overlaid on top of the overlaid buffer solutions in the ultracentrifuge tube, and was then subjected to a first sucrose density gradient ultracentrifugation at 10°C,

with a centrifugal force of 100,000 g, for 12 hours, using a Beckman ultrasonic centrifuge.

The 120NA120(+) was recovered in a portion with a sucrose concentration of about 30 to 40%.

The 120NA120(+) containing fraction recovered by the first sucrose density gradient ultracentrifugation was purified by Superdex 200 (gel filtration column) (made by Pharmacia Co., Ltd.) which was equilibrated with a buffer solution (0.3 M NaCl, 0.3% OTG, 50 mM glycine-NaOH, pH 10.0), whereby 120NA120(+) with a molecular weight of about 700 to 1000 kDa was recovered.

A 50%-sucrose concentration buffer solution was prepared by adding sucrose to a buffer solution (50 mM tris-HCl, pH 8.0, 20% ethanol) in such a manner that the concentration of sucrose in the buffer solution was 50%. In the same manner as mentioned above, a 20%-sucrose concentration buffer solution was prepared.

These buffer solutions were overlaid in an ultracentrifuge tube in the direction from the bottom to the top portion of the tube in the order of the 50%-sucrose concentration buffer solution and the 20%-sucrose concentration buffer solution.

The above-mentioned 120NA120(+) with a molecular weight of about 700 to 1000 kDa was overlaid on top of the overlaid buffer solutions in the ultracentrifuge tube, and was then subjected to a second sucrose density gradient centrifugation at 10°C, with a centrifugal force

of 100,000 g, for 12 hours, using the Beckman ultrasonic centrifuge, whereby the 120NA120(+) was concentrated and purified.

#### **Example 10**

##### **[Rearrangement of 120NA120 to 120NA120(+)]**

The OD 260/280 nm ratio of the 120NA120 purified in Example 9 was about 0.7.

To the purified 120NA120, there was added a purified DNA (about 1.3 to 0.7 Kbp) (made by Sigma Co., Ltd.), which was obtained from calf thymus and was subjected to sufficient cleavage by a restriction endonuclease Hae3. Furthermore, 6M urea, 20% sucrose and 1.0 M NaCl were added thereto.

This mixture was dialyzed against a buffer (50 mM tris-HCl, 0.3 M NaCl) at 4°C, whereby the 120NA120 was rearranged to a soluble 120NA120(+).

The soluble 120NA120(+) was purified by Superdex 200 (gel filtration column) (made by Pharmacia Co., Ltd.), whereby a purified 120NA120(+) was recovered in a portion with a molecular weight of about 700 to 1000 kDa. The OD 260/280 nm ratio of the thus recovered rearranged 120NA120(+) was about 1.8.

#### **Example 11**

##### **[Construction of Transformed *Escherichia coli* BL21 (DE3)/pW6A47C2NA for Expressing Nucleic Acid-Binding TP47]**

(TP47C2NA)]

A DNA fragment for coding a 47 kDa antigen derived from TP (*Treponema pallidum*), with Sequence ID No. 13 in the attached sequence table, was amplified by the PCR method, using as a template molecule a plasmid pW6A47C2 with a DNA fragment including a TP47kDa antigen region being introduced, and was then digested with a restriction endonuclease EcoRI and a restriction endonuclease BamH.

A TP47kDa antigen region-including DNA fragment 1.3 Kbp was separated by 1% agarose gel electrophoresis. This DNA fragment was inserted into an EcoRI-BamHI site of the expression plasmid pW6A shown in Fig. 1, whereby a plasmid pW6A47C2 (EcoRI/BamHI) was prepared.

A DNA fragment for coding a HBc nucleic acid-binding motif with Sequence ID No. 1 in the attached sequence table was amplified by the PCR method, using as a template molecule a plasmid pHBV-11, and was then digested with a restriction endonuclease BamHI and a restriction endonuclease HindIII.

A nucleic acid-binding motif-containing DNA fragment 110 bp was then separated by 1% agarose gel electrophoresis. This DNA fragment was inserted into a BamHI-HindIII site of the above plasmid pW6A47C2 (EcoRI/BamHI).

By use of this plasmid, *Escherichia coli* BL21 (DE3) was subjected to transformation, so that an ampicillin-

resistant transformed *Escherichia coli* BL21 (DE3)/pW6A47C2NA for expressing a nucleic acid-binding TP47 (hereinafter referred to as TP47C2NA) was obtained.

## Example 12

### [Purification of Insoluble TP47C2NA]

In the same manner as in Reference Example 2, the transformed *Escherichia coli* BL21 (DE3)/pW6ATP47C2NA prepared in Example 11 was cultured overnight in an LB culture medium at 37°C. The optical density (OD) of the culture medium was adjusted by preculture so as to have about 0.7 when measured with light with a wavelength of 600 nm. Expression induction was then carried out with the addition of 0.5 mM IPTG thereto, and the cultivation was continued for two hours and 30 minutes.

The *Escherichia coli* cultivation medium was then centrifuged, whereby the *Escherichia coli* was collected. To the thus collected *Escherichia coli*, 150 ml of a buffer solution (50 mM tris-HCl, pH 8.0, 20% ethanol) was added, and the mixture was ice-cooled and subjected to ultrasonic disruption.

This mixture was then centrifuged, whereby an expressed TP47C2NA was obtained as a soluble fraction as well as an insoluble fraction. The insoluble TP47C2NA fraction was made soluble by a buffer solution (6M urea,



50 mM tris-HCl, pH 8.0) and was then subjected to centrifugation, whereby a supernatant fraction was obtained.

The thus obtained supernatant fraction was subjected to ion exchange purification, using an SFF cationic ion exchange column (made by Pharmacia Co., Ltd.) which was equilibrated with a buffer solution (8M urea, sodium acetate, pH 6.0), with sodium chloride elution. TP47C2NA was recovered in an about 0.5M sodium chloride elution fraction.

The SFF eluted fraction was then purified, using Superdex 200 (gel filtration column) (made by Pharmacia Co., Ltd.) which was equilibrated with a buffer solution (6M urea-0.5M NaCl, 50 mM tris-HCl, pH 9.6). Thus, a purified TP47C2NA was obtained in a portion with a molecular weight of about 100 kDa.

The nucleotide sequence and the amino acid sequence of the TP47C2NA are respectively shown with Sequence ID No. 15 and Sequence ID No. 16 in the attached sequence table.

### **Example 13**

#### **[Purification of Soluble Nucleic Acid-bound TP47C2NA (TP47C2NA(+))]**

In the same manner as in Example 3, the *Escherichia coli* BL21 (DE3)/pW6ATP47C2NA prepared in Example 11 was

cultured overnight in an LB culture medium at 37°C. The optical density (OD) of the culture medium was adjusted by preculture so as to be about 0.7 when measured with light with a wavelength of 600 nm. Expression induction was then carried out with the addition of 0.5 mM IPTG thereto, and thereafter the cultivation was continued for two hours and 30 minutes.

The *Escherichia coli* cultivation medium was centrifuged, whereby the *Escherichia coli* was collected. To the thus collected *Escherichia coli*, 150 ml of a buffer solution (50 mM tris-HCl, pH 8.0, 20% ethanol, 0.3% OTG) was added, and the mixture was ice-cooled and subjected to ultrasonic disruption.

This mixture was then centrifuged, whereby a soluble nucleic acid-bound TP47C2NA (hereinafter referred to as "TP47C2NA(+)") was recovered.

A 50%-sucrose concentration buffer solution was prepared by adding sucrose to a buffer solution (50 mM tris-HCl, pH 8.0, 20% ethanol) in such a manner that the concentration of sucrose in the buffer solution was 50%. In the same manner as mentioned above, a 30%-sucrose concentration buffer solution, and a 20%-sucrose concentration buffer solution were prepared.

These buffer solutions were overlaid in an ultracentrifuge tube in the direction from the bottom to the top portion of the tube in the order of the 50%-

sucrose concentration buffer solution, the 30%-sucrose concentration buffer solution and the 20%-sucrose concentration buffer solution.

The TP47C2NA(+) containing soluble fraction was overlaid on top of the overlaid buffer solutions in the ultracentrifuge tube, and was then subjected to a first sucrose density gradient ultracentrifugation at 10°C, with a centrifugal force of 100,000 g, for 12 hours, using a Beckman ultrasonic centrifuge.

The TP47C2NA(+) was recovered in a portion with a sucrose concentration of about 30 to 45%.

The TP47C2NA(+) containing fraction recovered by the first sucrose density gradient ultracentrifugation was purified by Superdex 200 (gel filtration column) (made by Pharmacia Co., Ltd.) which was equilibrated with a buffer solution (0.3 M NaCl, 0.3% OTG, 50 mM glycine-NaOH, pH 10.0), whereby TP47C2NA(+) was recovered in a portion with a molecular weight of about 700 to 1000 kDa.

A 50%-sucrose concentration buffer solution was prepared by adding sucrose to a buffer solution (50 mM tris-HCl, pH 8.0, 20% ethanol) in such a manner that the concentration of sucrose in the buffer solution was 50%. In the same manner as mentioned above, a 20%-sucrose concentration buffer solution was prepared.

These buffer solutions were overlaid in an ultracentrifuge tube in the direction from the bottom to the top portion of the tube in the order of the 50%-

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sucrose concentration buffer solution and the 20%-sucrose concentration buffer solution.

The above-mentioned TP47C2NA(+) with a molecular weight of about 700 to 1000 kDa was overlaid on top of the overlaid buffer solutions in the ultracentrifuge tube, and was then subjected to a second sucrose density gradient centrifugation at 10°C, with a centrifugal force of 100,000 g, for 12 hours, using the Beckman ultrasonic centrifuge, whereby the TP47C2NA(+) was concentrated and purified.

#### Example 14

##### [Rearrangement of TP47C2NA to TP47C2NA(+)]

The OD 260/280 nm ratio of the TP47C2NA purified in Example 12 was about 0.6.

To the purified TP47C2NA, there was added a purified DNA (about 1.3 to 0.7 Kbp) (made by Sigma Co., Ltd.), which was obtained from calf thymus and was subjected to sufficient cleavage by a restriction endonuclease Hae3. Furthermore, 6M urea, 20% sucrose and 1.0 M NaCl were added thereto.

This mixture was dialyzed against a buffer (50 mM tris-HCl, 0.3 M NaCl) at 4°C, whereby the TP47C2NA was rearranged to a soluble TP47C2NA(+).

The soluble TP47C2NA(+) was purified by Superdex 200 (gel filtration column) (made by Pharmacia Co., Ltd.),

whereby a purified 120NA120(+) was recovered in a portion with a molecular weight of about 700 to 1000 kDa. The OD 260/280 nm ratio of the thus recovered rearranged TP47C2NA(+) was about 1.8.

#### Example 15

[Construction of Transformed *Escherichia coli* BL21 (DE3)/pW6ACV Core 120pro1 for Expressing Mouse Protamine 1 fused 120 (120pro1)]

A DNA fragment for coding a mouse protamine 1 with Sequence ID No. 17 in the attached sequence table was isolated, and amplified by the PCR method, using as a template molecule a mouse protamine 1 cDNA, and was then digested with a restriction endonuclease EcoRI and a restriction endonuclease BamH.

A mouse protamine 1 region-including DNA fragment 160 bp was separated by 1% agarose gel electrophoresis. This DNA fragment was inserted into an EcoRI-BamHI site of the plasmid pW6AHCV core 120 (NheI/EcoRI) prepared in Example 1.

By use of this plasmid, *Escherichia coli* BL21 (DE3) was subjected to transformation, so that an ampicillin-resistant transformed *Escherichia coli* BL21 (DE3)/pW6ACV core 120pro1 for expressing a mouse protamine 1 fused 120 (hereinafter referred to as 120pro1) was obtained.

#### Example 16

[Purification of 120pro1]

In the same manner as in Reference Example 2, the transformed *Escherichia coli* BL21 (DE3)/pW6AHCV core 120pro1 prepared in Example 15 was cultured overnight in an LB culture medium at 37°C. The optical density (OD) of the culture medium was adjusted by preculture so as to have about 0.7 when measured with light with a wavelength of 600 nm. Expression induction was then carried out with the addition of 0.5 mM IPTG thereto, and the cultivation was continued for 2 hours and 30 minutes.

The *Escherichia coli* cultivation medium was then centrifuged, whereby the *Escherichia coli* was collected. To the thus collected *Escherichia coli*, 150 ml of a buffer solution (50 mM tris-HCl, pH 8.0, 20% ethanol) was added, and the mixture was ice-cooled and subjected to ultrasonic disruption.

This mixture was then centrifuged, whereby an expressed 120pro1 was obtained as a soluble fraction as well as an insoluble fraction. The insoluble 120pro1 fraction was made soluble by a buffer solution (6M urea, 50 mM glycine-NaOH, pH 11.0) and was then subjected to centrifugation, whereby a supernatant fraction was obtained.

The thus obtained supernatant fraction was subjected to ion exchange purification, using an SFF cationic ion exchange column (made by Pharmacia Co., Ltd.) which was

equilibrated with a buffer solution (6M urea-glycine-NaOH, pH 11.0), with sodium chloride elution. 120pro1 was recovered in an about 0.5M sodium chloride elution fraction.

The SFF eluted fraction was then purified, using Superdex 200 (gel filtration column) (made by Pharmacia Co., Ltd.) which was equilibrated with a buffer solution (6M urea-0.5M NaCl, 50 mM tris-HCl, pH 9.6). Thus, a purified 120pro1 was obtained in a portion with a molecular weight of about 22 kDa.

The nucleotide sequence and the amino acid sequence of the 120pro1 are respectively shown with Sequence ID No. 19 and Sequence ID No. 20 in the attached sequence table.

#### **Example 17**

##### **[Rearrangement of 120pro1 to 120pro1(+)]**

The OD 260/280 nm ratio of the 120pro1 purified in Example 16 was about 0.7.

To the purified 120pro1, there was added a purified DNA (about 1.3 to 0.7 Kbp) (made by Sigma Co., Ltd.), which was obtained from calf thymus and was subjected to sufficient cleavage by a restriction endonuclease Hae3. Furthermore, 6M urea, 20% sucrose and 1.0 M NaCl were added thereto.

This mixture was dialyzed against a buffer (50 mM

tris-HCl, 0.3M NaCl) at 4°C, whereby the 120pro1 was rearranged to a soluble 120pro1(+).

The soluble 120pro1(+) was purified by Superdex 200 (gel filtration column) (made by Pharmacia Co., Ltd.), whereby a purified 120pro1(+) was recovered in a portion with a molecular weight of about 700 to 1000 kDa. The OD 260/280 nm ratio of the thus recovered rearranged 120pro1(+) was about 1.7.

Thus, the present invention provides the nucleic acid-bound polypeptide with various properties of the polypeptide being changed, without changing the antigenicity thereof. The use of the nucleic acid-bound polypeptide of the present invention makes it possible to perform immunoassays which have been conventionally impossible.

Furthermore, according to the present invention, there is provided a method of recovering a genetic product in a soluble fraction, which has conventionally been recovered in an insoluble fraction.



Japanese Patent Application No. 8-134444 filed May 1,  
1996, is hereby incorporated by reference.

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WHAT IS CLAIMED IS:

1. A nucleic acid-bound polypeptide which is produced by binding a nucleic acid to a polypeptide.

2. The nucleic acid-bound polypeptide as claimed in Claim 1, wherein said nucleic acid is bound to at least one terminus of said polypeptide.

3. The nucleic acid-bound polypeptide as claimed in Claim 1, further comprising a nucleic acid-binding motif through which said nucleic acid is bound to said polypeptide.

4. The nucleic acid-bound polypeptide as claimed in Claim 2, further comprising a nucleic acid-binding motif through which said nucleic acid is bound to at least said one terminus of said polypeptide.

5. The nucleic acid-bound polypeptide as claimed in Claim 3, wherein said polypeptide and said nucleic acid-binding motif are expressed in the form of a fusion polypeptide by genetic engineering.

6. The nucleic acid-bound polypeptide as claimed in Claim 4, wherein said polypeptide and said nucleic acid-binding motif are expressed in the form of a fusion polypeptide by genetic engineering.

Figure 1 consists of six sub-diagrams labeled (a) through (f), arranged vertically. Each diagram shows a cross-section of a fluid with two vortices. The vortices are represented by shaded regions with a central core. The diagrams illustrate the evolution of the vortices over time. (a) shows the initial state with two distinct vortices. (b) through (f) show the vortices at successive time steps, showing their interaction and the development of secondary vortices. The diagrams are labeled with time values: (a) 0.0, (b) 0.2, (c) 0.4, (d) 0.6, (e) 0.8, and (f) 1.0. The vortices are shown in a coordinate system with x and y axes. The x-axis is horizontal and the y-axis is vertical. The vortices are located in the upper half of the domain. The diagrams show the vortices moving and interacting, with the formation of secondary vortices and the eventual merging of the primary vortices.

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10. The nucleic acid-bound polypeptide as claimed in Claim 2, wherein said polypeptide is an antigen to be assayed by an immunoassay.

11. The nucleic acid-bound polypeptide as claimed in Claim 3, wherein said polypeptide is an antigen to be assayed by an immunoassay.

12. The nucleic acid-bound polypeptide as claimed in Claim 4, wherein said polypeptide is an antigen to be assayed by an immunoassay.

13. The nucleic acid-bound polypeptide as claimed in Claim 5, wherein said polypeptide is an antigen to be assayed by an immunoassay.

14. The nucleic acid-bound polypeptide as claimed in Claim 6, wherein said polypeptide is an antigen to be assayed by an immunoassay.

15. A method of producing a nucleic acid-bound polypeptide comprising the steps of:

producing a polypeptide by genetic engineering,

binding a nucleic acid to said polypeptide to produce a nucleic acid-bound polypeptide as a soluble fraction, and

purifying said nucleic acid-bound polypeptide from said soluble fraction.

16. The method of producing said nucleic acid-bound polypeptide as claimed in Claim 15, wherein the step of binding said nucleic acid to said polypeptide to produce said nucleic acid-bound polypeptide comprises the steps of:

fusing a gene which codes said polypeptide and a gene which codes a nucleic acid-binding motif for binding said nucleic acid to said polypeptide to produce a fusion gene, and

expressing said fusion gene to produce said nucleic

acid-bound polypeptide via said nucleic acid-binding motif.

17. An immunoassay for assaying an antigen comprising a polypeptide, or an antibody corresponding to said polypeptide, using as an antigen for said immunoassay a nucleic acid-bound polypeptide obtainable by binding a nucleic acid to said polypeptide.

18. The immunoassay as claimed in Claim 17, wherein said nucleic acid is bound to at least one terminus of said polypeptide.

19. The immunoassay as claimed in Claim 17, wherein said nucleic acid-bound polypeptide further comprises a nucleic acid-binding motif through which said nucleic acid is bound to said polypeptide.

20. The immunoassay as claimed in Claim 18, wherein said nucleic acid-bound polypeptide further comprises a nucleic acid binding motif through which said nucleic acid is combined with at least one terminus of said polypeptide.

21. The immunoassay as claimed in Claim 19, wherein said polypeptide and said nucleic acid-binding motif are expressed in the form of a fusion polypeptide by genetic

engineering.

22. The immunoassay as claimed in Claim 20, wherein said polypeptide and said nucleic acid binding motif are expressed in the form of a fusion polypeptide by genetic engineering.

23. The immunoassay as claimed in Claim 19, wherein said nucleic acid-binding motif has an amino acid sequence with sequence No. 2 defined in a sequence table attached to the specification of this application.

24. The immunoassay as claimed in Claim 20, wherein said nucleic acid-binding motif has an amino acid sequence with sequence No. 2 defined in a sequence table attached to the specification of this application.

25. An agglutination immunoassay for assaying an antigen comprising a polypeptide, or an antibody corresponding to said antigen, using as said antigen a nucleic acid-bound polypeptide which is produced by binding a nucleic acid to a polypeptide, and fixing said nucleic acid-bound polypeptide on the surface of particles.

26. The agglutination immunoassay as claimed in Claim 25, wherein said nucleic acid is bound to at least

one terminus of said polypeptide.

27. The agglutination immunoassay as claimed in Claim 25, wherein said nucleic acid-bound polypeptide further comprises a nucleic acid-binding motif through which said nucleic acid is bound to said polypeptide.

28. The agglutination immunoassay as claimed in Claim 26, wherein said nucleic acid-bound polypeptide further comprises a nucleic acid-binding motif through which said nucleic acid is bound to at least one terminus of said polypeptide.

29. The agglutination immunoassay as claimed in Claim 27, wherein said polypeptide and said nucleic acid-binding motif are expressed in the form of a fusion polypeptide by genetic engineering.

30. The agglutination immunoassay as claimed in Claim 28, wherein said polypeptide and said nucleic acid binding motif are expressed in the form of a fusion polypeptide by genetic engineering.

31. The agglutination immunoassay as claimed in Claim 27, wherein said nucleic acid-binding motif has an amino acid sequence with sequence No. 2 defined in a sequence table attached to the specification of this

application.

32. The agglutination immunoassay as claimed in Claim 28, wherein said nucleic acid-binding motif has an amino acid sequence with sequence No. 2 defined in a sequence table attached to the specification of this application.

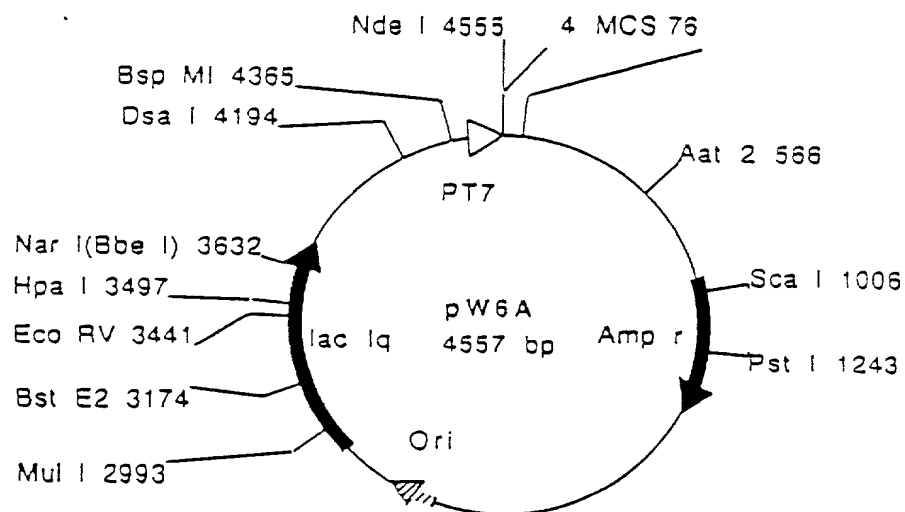


ABSTRACT OF THE DISCLOSURE

A nucleic acid-bound polypeptide produced by binding a nucleic acid to a polypeptide, a method of producing the nucleic acid-bound polypeptide, and applications of the nucleic acid-bound polypeptide, including immunoassays for an antigen or antibody, such as an agglutination immunoassay are provided.

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Fig. 1



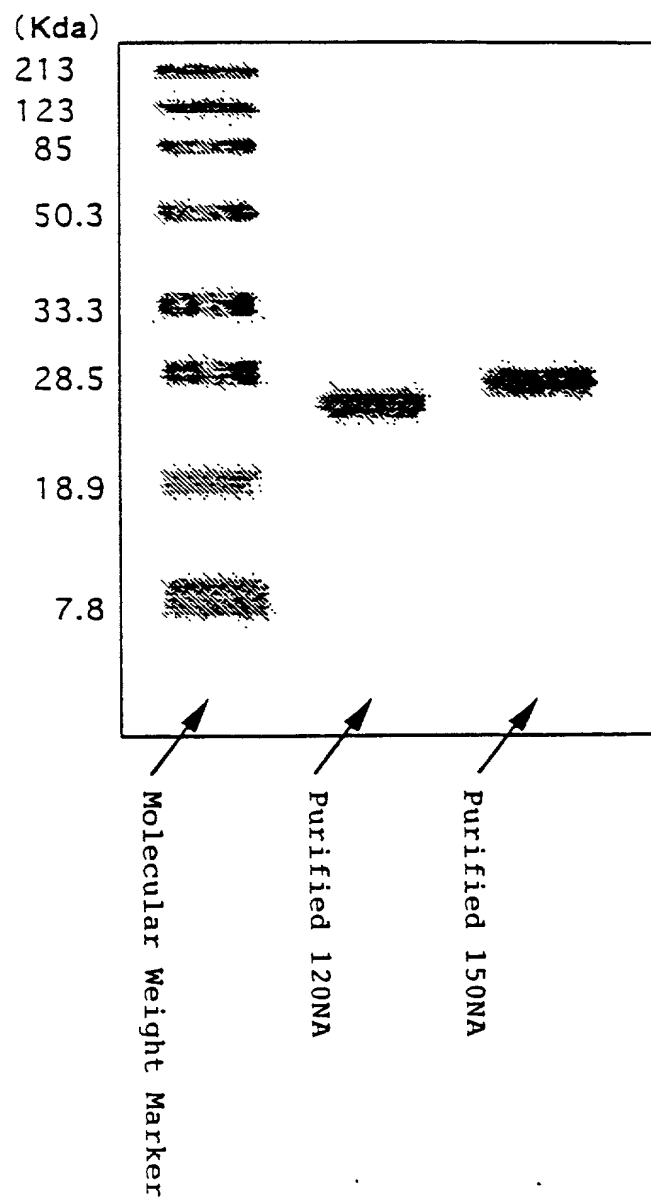
4475                      4495                      0  
 /                      /                      /  
TAATACGACTCACTATATGGAATTGTGAGCGGATAACAATT.....CATATG  
 T7 promoter                      lac operator                      Nde I

| Nhe I | | Eco RI | | Sal I | | Xho I | | Bam HI | | <sup>\*</sup>Apa I | | Xba I |  
 MCS: ATG GCT AGC GAA TTC GTC GAC CTC GAG GGA TCC GGG CCC TCT AGA  
 start codon  
 | Not I |                      Eco T22 I                      Kpn I |                      Hind 3 |  
 TGC GGC CGC ATG CAT GGT ACC TAA CTA ACT AAG CTT  
                     Eag I                      TL term

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Fig. 2

Western Blot



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# Declaration, Power Of Attorney and Petition

Page 1 of 3

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

NUCLEIC ACID-BOUND POLYPEPTIDE, METHOD OF PRODUCING NUCLEIC  
ACID-BOUND POLYPEPTIDE, AND IMMUNOASSAY USING THE POLYPEPTIDE

the specification of which

☐ is attached hereto.

☒ was filed on April 30, 1997 as

Application Serial No. 08/841,657

and amended on \_\_\_\_\_.

☐ was filed as PCT international application

Number \_\_\_\_\_

on \_\_\_\_\_,

and was amended under PCT Article 19

on \_\_\_\_\_ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	Country	Day/Month/Year	Priority Claimed
<u>8-134444</u>	<u>JAPAN</u>	<u>1/MAY/1996</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No

We (I) hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

_____	_____
(Application Number)	(Filing Date)
_____	_____
(Application Number)	(Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	Filing Date	Status (pending, patented, abandoned)
_____	_____	_____
_____	_____	_____
_____	_____	_____

And we (I) hereby appoint: Norman F. Oblon, Registration Number 24,618; Marvin J. Spivak, Registration Number 24,913; C. Irvin McClelland, Registration Number 21,124; Gregory J. Maier, Registration Number 25,599; Arthur I. Neustadt, Registration Number 24,854; Richard D. Kelly, Registration Number 27,757; James D. Hamilton, Registration Number 28,421; Eckhard H. Kuesters, Registration Number 28,870; Robert T. Pous, Registration Number 29,099; Charles L. Gholz, Registration Number 26,395; Vincent J. Sunderdick, Registration Number 29,004; William E. Beaumont, Registration Number 30,996; Steven B. Kelber, Registration Number 30,073; Robert F. Gnuse, Registration Number 27,295; Jean-Paul Lavalleye, Registration Number 31,451; Timothy R. Schwartz, Registration Number 32,171; Stephen G. Baxter, Registration Number 32,884; Martin M. Zoltick, Registration Number 35,745; Robert W. Hahl, Registration Number 33,893; Richard L. Treanor, Registration Number 36,379; Steven P. Weihrouch, Registration Number 32,829; John T. Goolkasian, Registration Number 26,142; Marc R. Labgold, Registration Number 34,651; William J. Healey, Registration Number 36,160; Richard L. Chinn, Registration Number 34,305; Steven E. Lipman, Registration Number 30,011; and Jacques M. Dulin, Registration Number 24,067; our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to the firm of OBLON, SPIVAK, McCLELLAND, MAIER & NEUSTADT, P.C., whose Post Office Address is: Fourth Floor, 1755 Jefferson Davis Highway, Arlington, Virginia 22202.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Fuminori TAKEMURA  
NAME OF FIRST ~~SOLE~~ INVENTOR

*Fuminori Takemura*  
Signature of Inventor

Residence: #806, Sakuragaoka Danchi  
22, 3-44-13, Sakuragaoka,  
Higashiyamato-shi, Tokyo, Japan  
Citizen of: Japan  
Post Office Address: same as above


May 8, 1997  
Date

Eiichi UENO  
NAME OF SECOND JOINT INVENTOR

  
Signature of Inventor

May 8, 1997  
Date

Satoru ITOH  
NAME OF THIRD JOINT INVENTOR

  
Signature of Inventor

May 8, 1997  
Date

NAME OF FOURTH JOINT INVENTOR

Signature of Inventor

Date

NAME OF FIFTH JOINT INVENTOR

Signature of Inventor

Date

Residence: #501, Kurio Hino san-bankan,  
6-24-3, Tamadaira, Hino-shi,  
Tokyo, Japan

Citizen of: Japan

Post Office Address: same as above

Residence: #I-101, Royal Mezon Minami-  
naruse, 1-18-1, Narusegaoka,  
Machida-shi, Tokyo, Japan

Citizen of: Japan

Post Office Address: same as above

Residence:

Citizen of:

Post Office Address:

Residence:

Citizen of:

Post Office Address: